II. REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and the reasons that follow.

Claim Amendments

Claims 5-9, 16-17, 24-25, 32-33, and 40-55 stand canceled without prejudice or disclaimer.

Claims 12, 20, 28, and 36 are now canceled without prejudice or disclaimer.

Claims 10-11, 15, 18-19, 23, 26-27, 31, 34-35, and 39 stand withdrawn as being drawn to non-elected invention.

Claims 1-4,13-14, 21-22, 29-30, and 37-38 currently are amended. No new matter is added by these amendments. Support for amending Claims 1-4 can be found in Claims 12, 20, 28, and 36 as originally filed and now cancelled. Support for adding the dose of B7.1 "from 60-180 μg of a deoxyribonucleic acid (DNA) encoding B7.1" to Claims 1-2, and 4 can be found, for example, on page 4, lines 13-36; page 14, lines 7-8 and on page 18, lines 25-28 of application as filed. Support for adding the phrase "optimal dose" in Claim 3 can be found, for example, page 18, lines 19-34 of the application as filed. Claims 14, 22, 30, and 38 has been amended to recite the transition term "comprises" rather than the informal term "includes".

Claims 13, 21, 29, and 37 currently are amended to correct the claim dependencies and the antecedent basis.

New claims 56-58 are added. No new matter is added by the new claims. Support for the new claims can be found, for example, on page 4, lines 13 to 36; page 10, lines 16-26; and page 14, lines 10-11 of the application as filed.

The cancellation and/or amendment of the subject matter is not intended to be a dedication of the subject matter to the public. Applicant reserves the right to file one or more continuation, divisional, or continuation-in-part application to the cancelled subject matter.

After amending the claims as set forth above, Claims 1-4, 13-14, 21-22, 29-30, 37-38, and 56-58 are under consideration.

Entry of the amendment is respectfully requested. The amendments are made in a sincere effort to place the claims in condition for allowance or in better form for consideration on appeal and do not require an additional search of the art. The amendments were not made earlier as it was Applicant's belief that the claims as previously presented defined patentable subject matter.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Claim Rejection Under 35 U.S.C. §103(a)

Claims 1-4, 12-14, 20-22, 28-30, 36-38, and 44-46 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Futami *et al.* (*J. of Immunotherapy*, vol. 12, 247-255, 1992) in view of Wilson *et al.* (*Int. J. Radiation Oncology Biol. Phys.*, vol. 42, 905-908, 1998) and in view of Olsson *et al.* (*Int. Immunology*, vol. 10, 499-506, 1998). The Office alleged that Futami *et al.* teach a method of treating tumor by administering 5-methyl XAA in conjunction with the T-cell stimulating molecule IL-2. Wilson *et al.* is cited for teaching that DMXAA potentiates tumor radiation response compared to each treatment alone. Olsson *et al.* is cited for teaching that human IL-2 production is induced by CD80 (B7.1, a CAM molecule) in cancer cells and T cells. The Office further alleged that it in view of the above separate teachings of the prior art, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the claimed invention was made to replace the IL-2 with its stimulator (CAM) and XAA with its analogue, DMXAA. *See* pages 3-4 of the Office Action.

Applicant traverses the rejection for the following reasons.

After application of the asserted teachings of the prior art and the facts of Applicant's invention, Applicant submits that the claimed invention is not obvious over the combination of the teachings of the cited art because the cited art failed to teach or appreciate that administration

of B7.1 prior to DMXAA would potential the activity of DMXAA and B7.1. Stated another way, the combination of DMXAA and B7.1 and the order of administration provides a synergistic response that was not taught, suggested or expected from the teachings of the prior art.

In a sincere effort to expedite prosecution and more clearly and distinctly claim the invention, Applicant has amended the claims to recite that the invention requires administering a certain suboptimal dose of a DNA expressing B7.1 (i.e., from 60-180 μg), prior to administering DMXAA to a mammal or patient suffering from cancer or a large or advanced tumors.[1] Claims 13, 21, and 37 recite that the DNA encoding B7.1 be administered 12 to 48 hours prior to administration of DMXAA.

<u>Cited References Do Not Teach Claimed Invention</u>

Turning now to the teachings of the cited art. Futami *et al.* teach the administration of XAA, not DMXAA one day <u>before</u> administration of rhIL-2 (*see* Futami *et al.*, p.249, ¶ 1). Indeed, Futami *et al.* teach that prior administration of XAA <u>is necessary</u> to induce cytokine mRNA. Futami *et al.* disclose that:

> "[t]he therapeutic effects of FAA may be related to its ability to induce cytokines that synergize with IL-2 (9). Since the XAA derivatives tested in this study are structurally similar to FAA, and also induce a similar pattern of cytokines, we hypothesized that these might also synergize with IL-2 for antitumor effects."

See page 251, right hand column, first paragraph.

Futami *et al.* teach that the cytokines induced by XAA synergize with IL-2 for alleged anti-tumor effects. Therefore, Futami *et al.* fail to teach or suggest Applicant's claimed agents, i.e., DMXAA and B7.1, or the claimed order of administration.

Olsson *et al.* use cancer cell lines to describe the effect of two different CAMs, CAM B7.1 and CAM B7.2. Nowhere do Olsson *et al.* teach treatment of cancer using B7.1 or administration of B7.1 in combination with DMXAA, let alone the order of administration or the amount of B7.1.

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[1] "advanced or large tumors" defined as >0.5 cm on p. 16, lines 12-14 of the application as filed.

Wilson *et al.* only disclose DMXAA as an anti-cancer agent. Wilson *et al.* do not teach combination of DMXAA with B7.1, prior administration of B7.1 to DMXAA or administering DNA encoding B7.1 in an amount in the range of 60-180 μg.

The Cited References Fail To Suggest The Combination

The combined teachings of the cited references do not negate patentability under 35 U.S.C. §103 as the combined teachings provide no motivation for combination as the Office has suggested and even if combined, one of skill in the art would not have an expectation of success when practicing the claimed invention.

Futami *et al.* do not motivate a person of ordinary skill in the art to substitute rhIL-2 with B7.1 and XAA with DMXAA to arrive at the components of the claimed invention. Further, Futami *et al.* do not motivate a person of ordinary skill in the art to reverse the order of administration and administer the DNA encoding B7.1 prior to DMXAA. Indeed, the mechanism of action of inducing cytokines by XAA warrants the need for administering XAA prior to rhIL-2 or B7.1 which stimulates IL-2 production.

Applicant brings to Office's attention, Larchain et al. *Clinical Cancer Research* (2000) 6:2913-2920 (**Exhibit I**). Larchain *et al.* clearly show that while a sequential administration of IL-2 followed by B7.1 significantly increased a systemic immune response that translated into increased survival, the administration of B7.1 alone; IL-2 alone; or B7.1 followed by IL-2 did not prove efficacious. *See* page 2913, abstract, right hand column and page 2918, right hand column. Larchain *et al.* clearly show not only that B7.1 cannot be substituted by IL-2 but also that the order of administration of IL-2 and B7.1 is a significant parameter for effective treatment.

Therefore, a person of ordinary skill in the art would not be motivated to modify the teachings of the primary reference Futami et al. with the teachings of the secondary references as suggested by the Office. Even assuming *arguendo* such a suggestion existed, which Applicant maintains it does not, the cited art provides no reasonable expectation of success to substitute XAA with DMXAA, substitute rhIL-2 with B7.1 and then reverse the order of administration, to arrive at the claimed invention.

Olsson *et al.* fail to fulfill the deficiencies of Futami *et al.* Olsson *et al.* do not teach or suggest administering DNA encoding B7.1 in a combination therapy with DMXAA or to administer DNA encoding B7.1 prior to DMXAA. Therefore, in the absence of any suggestion or motivation in Olsson *et al.*, there is neither reason to combine the teachings of the cited art nor a reasonable expectation of success for a person of ordinary skill in the art to use B7.1 in combination with DMXAA for the treatment of cancer and administer the DNA expressing B7.1 prior to DMXAA in an amount in the range of 60-180 μg.

Wilson *et al.* only disclose DMXAA as an anti-cancer agent. Wilson *et al.* do not teach combination of DMXAA with B7.1, prior administration of DNA encoding B7.1 to DMXAA or administering DNA encoding B7.1 in an amount in the range of 60-180 μg. There is no suggestion or motivation in Wilson *et al.* to combine DMXAA with B7.1.

The Office states[2] that, it would be *prima facie* obvious to administer one reagent prior to another. Applicant contends that the Office has made an unsupported statement and request the Office to show evidence to support the statement.

Applicant submits that in the absence of any teaching, suggestion, or motivation in either of the cited references, a person of skill in the art will not be motivated to combine the reference teachings and come up with the claimed invention.

Claimed Methods Provide Unexpected Results

The amended claims are directed to administration of DNA encoding B7.1 prior to (in one aspect 12 to 48 hours) the administration of the tumor growth restricting agent DMXAA. Applicant's specification shows[3] that when the agents are timed in the specifically claimed order of administration, the combined therapy eradicates large tumors:

> … established tumours (0.6-0.8 cm in diameter) were first treated with B7.1 to stimulate anti-tumour immunity, and DMXAA and FAA were administered one day later to retard tumour growth. Remarkably, tumours rapidly diminished in response to the combination

[2] Office Action dated 08/23/2006, p. 8, end of ¶ 2

[3] p. 16, lines 25-34 to p. 17, lines 1-7 of the application as filed.

> of B7.1 and DMXAA accompanied by massive necrosis, such that by the third week of treatment tumours had completely disappeared (FIG. 1b).

Applicant has demonstrated that prior administration of B7.1 stimulates anti-tumour immunity which by subsequent administration of DMXAA retards tumor growth. Applicant notes that the specification discloses that the tumor growth restricting agent acts by reducing blood flow to tumors including inhibiting or preventing angiogenesis. *See* page 2, lines 31-33 of the application as filed. Applicant further notes that the specification also discloses that the prior administration of DMXAA may result in dying and necrotic tumor cells which may not be able to adequately express B7.1 and might impair CAM-mediated anti-tumor immunity. *See* page 16, lines 25-29 of the application as filed. Therefore, the prior administration of B7.1 to DMXAA is required for the combination therapy to be effective.

Quite unexpectedly, the order of administration also eliminates the high gene dosage effect of B7.1 administration. Applicant's specification discloses on page 18, lines 19-34:

> Further, we have previously reported that B7.1 and other costimulatory CAMs display a restrictive gene dosage effect, such that gene transfer of 60 μg of B7.1/pCDM8 expression plasmid is optimal, whereas lesser or greater amounts are much less effective (Kanwar et al, 1999). To investigate whether a high gene dosage would impair combination therapy, tumours were injected with varying amounts (90-180 μg) of B7.1/pCDM8 plasmid followed by administration of an optimal dose of DMXAA (25 mg/Kg) (FIG. 4a). All mice rapidly rejected their tumours, and a rechallenge of $2x10^5$ parental EL-4 cells. Thus an identical outcome is achieved with a broad high dose range of the therapeutic gene and an optimal dose of DMXAA. In contrast, when the DMXAA concentration was suboptimal, a gene dosage effect is clearly evident, such that only large amounts (180 μg) of B7.1/pCDM8 expression plasmid could generate effective anti-tumour immunity (FIG. 4b).

Therefore, the instant application shows surprising results that an optimal dose of DMXAA results in an effective therapy even with a low dosage of gene as well as high dosage of gene. This is in contrast to Futami *et al.* where the dose of the XAA analogue is close to the maximum tolerated dose (*see* for example, page 249, right hand column, last paragraph to page 250, left hand column, ¶1) used in combination with 30,000U of rhIL-2 (*see* for example, Table 2, and Figs. 2-4). Firstly, since rhIL-2 in Futami *et al.* is a protein whereas Applicant administers DNA encoding B7.1, there can be no direct comparison of 30,000U of rhIL-2 used in Futami *et*

al. vis a vis 60-180 μg of DNA encoding B7.1 used in the claimed invention. Secondly, instant application do not disclose induction of IL-2 by B7.1, instead hypothesizes generation of cytotoxic T lymphocytes. *See* page 19 of the application as filed. Finally, the induction of IL-2 by B7.1, if any, is not quantitative. Therefore, a person of ordinary skill in the art cannot deduce from Futami *et al.* to substitute rhIL-2 with DNA expressing B7.1 and administer it in an amount of 60-180 μg, as in the claimed invention.

In sum, in the absence of any teaching, suggestion or motivation in Futami *et al.*, or the secondary references, a person of ordinary skill in the art will not be motivated to administer DNA expressing B7.1 before the administration of tumor growth restricting agent. There is no reasonable expectation of success to:

a) substitute the XAA analogue of Futami *et al.* with DMXAA;

b) substitute rhIL-2 with DNA encoding B7.1;

c) substitute 30,000U of rhIL-2 with 60-180 μg of DNA encoding B7.1;

c) substitute small tumors of Futami *et al.* with large or advanced tumors; and

d) reverse the order of administration of Futami *et al.*,

all of which are necessary to arrive at the claimed invention. For these reasons, the rejection of the amended claims under 35 U.S.C. §103(a) is improper and removal of the rejection is respectfully requested.

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

III. CONCLUSION

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or

credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date Oct. 17, 2008

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EXHIBIT I

Vol. 6, 2913–2920, July 2000

Effectiveness of Combined Interleukin 2 and B7.1 Vaccination Strategy Is Dependent on the Sequence and Order: A Liposome-mediated Gene Therapy Treatment for Bladder Cancer[1]

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ABSTRACT

We have developed a novel liposome-mediated immunogene therapy using interleukin 2 (IL-2) and B7.1 in a murine bladder cancer model. A carcinogen-induced murine bladder cancer cell line, MBT-2, was transfected with cationic liposome 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide/dioleolylphosphatidylethanolamine and IL-2 plasmid. The optimized transfection condition generated IL-2 levels of 245–305 ng/10^6 cells/24 h, 100-fold higher than the levels seen with retrovirus transfection. Ninety percent of the peak level of IL-2 production was maintained for up to 11 days after transfection. Animal studies were conducted in C3H/HeJ female mice with 2 × 10^4 MBT-2 cells implanted orthotopically on day 0. Multiple vaccination schedules were performed with i.p. injection of 5 × 10^6 IL-2 and/or B7.1 gene-modified cell preparations. The greatest impact on survival was observed with the day 5, 10, and 15 regimen. Control animals receiving retrovirally gene-modified MBT-2/IL-2 cell preparations had a median survival of 29 days. Animals receiving the IL-2 liposomally gene-modified cell preparation alone had a median survival of 46 days. Seventy-five percent of animals receiving IL-2 followed by B7.1 gene-modified tumor vaccines were the only group to show complete tumor-free survival at day 60. All of these surviving animals rejected the parental MBT-2 tumor rechallenge and survived at day 120 with a high CTL response. In conclusion, liposome-mediated transfection demonstrates a clear advantage as compared with the retroviral system in the MBT-2 model. Multi-agent as opposed to single-agent cytokine gene-modified tumor vaccines were beneficial. These "targeted" sequential vaccinations using IL-2 followed by B7.1 gene-modified tumor cells significantly increased a systemic immune response that translated into increased survival.

INTRODUCTION

The majority of human neoplasms are treated by the traditional modalities of surgery, radiation, or chemotherapy, either independently or in combination. The development of immunotherapeutic models over the past 15 years has led to multiple human protocols for the treatment of cancer (1). These models are predicated on the basic assumption that tumor-specific antigens exist and that the patient's immune system fails to either recognize or effectively respond to these antigens (2, 3). The goal of immunotherapy is therefore to increase tumor antigen recognition and enhance the antitumor response (4). In studies using the systemic administration of cytokines, such as IL-2,[3] profound inhibitory effects on tumor progression in animal models were seen, but only a limited therapeutic benefit was observed when IL-2 was administered to cancer patients (5–7). This limited efficacy was due in part to the toxicity resulting from the high doses of IL-2 required to stimulate an immune response in humans (8). Other studies using repeated injections of IL-2 directly into the tumor site have attempted to initiate the regression of established tumors or to induce immunological memory (9, 10). These alternative therapeutic options, although generating great interest, have met with sporadic clinical success to date (11).

A new form of immunomodulation using gene transfer techniques is now being actively investigated for a variety of malignancies (12). This treatment requires the insertion of a plasmid DNA encoding a cytokine gene directly into tumor cells. These "gene-modified" tumor cells then produce this cytokine, resulting in enhanced tumor antigen recognition and a documented increase in an antigen-specific immune response (13–15). We have previously investigated this new technique in the MBT-2 murine bladder cancer model (16, 17). The MBT-2 tumor was induced in C3H mice by oral administration of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide, a potent carcinogen that resulted in bladder neoplasms in 80–90% of animals

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[1] Supported by an American Foundation for Urologic Disease Fellowship and the Peter T. Joseph Foundation (W. A. L.).

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[3] The abbreviations used are: IL-2, interleukin 2; FBS, fetal bovine serum; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; DOPE, dioleolylphosphatidylethanolamine; APC, antigen-presenting cell.

(18). This poorly differentiated transitional cell carcinoma, which exhibits metastatic progression, resembles, both grossly and histologically, human transitional cell bladder cancer. In addition, treatment regimens that have been successful in this murine model have also shown efficacy in human bladder cancer (19, 20). In our previous research using retrovirus as the transfecting agent, cytokine genes including IL-2 and granulocyte macrophage colony-stimulating factor were successfully incorporated and stably expressed in MBT-2 tumor cells *in vitro* (16, 17). *In vivo* experiments showed that i.p. injection of these irradiated cytokine gene-modified cell preparations in tumor-bearing animals resulted in a significant survival advantage.

Although retroviral vectors provide genomic integration and a permanence of transduction, retroviral vector transfection is a time-consuming process and results in low expression of gene product (21). Additional limitations, including the safety issues of replication defects in this viral vector system and the fact that only dividing cells can be transduced, inhibit the potential of retroviral vectors as optimal transfecting agents for human application (22).

This study evaluates an alternative transduction mechanism using cationic liposomes. As reported previously by Vieweg *et al.* (23), liposome-mediated transfection has several advantages over viral vector systems in the use of promulgating gene-modified tumor cells. Liposome-mediated transfection has no infectious or little immunogenic potential, characteristics that are critical when considering human application (24, 25). Because the transfection is not based on genomic integration, gene product expression is transient (26). As opposed to the retroviral system, multiple copies of the plasmid can be transduced per cell, leading to higher production of the gene for a shorter period of time. We have hypothesized that if gene expression persists long enough for significant immunomodulation, the transient nature of the transfection becomes less important. Using an adeno-associated virus-based plasmid, we evaluated *in vitro* liposome-mediated transfection in the MBT-2 model. To establish conditions for optimal transfection, we studied four distinct parameters: (*a*) toxicity [liposomes have inherent lytic effects on mammalian cells when administered in high concentrations (27)]; (b) the critical liposome:DNA ratio producing the least toxicity with the highest gene expression; (*c*) the time of exposure to the liposome-DNA complex, *i.e.*, the least amount of transfection time to produce the greatest gene expression; and (*d*) the effect of growth factors, such as FBS, on the potential inhibition of liposome-mediated transfection.

Previous investigations studied the MBT-2 bladder cancer model *in vivo* by using the injection of MBT-2 cells either intradermally or s.c. to establish the tumor (28–30). Our laboratory devised an alternative method, *i.e.*, orthotopic implantation. This method involves the direct injection of MBT-2 cells into the bladder wall via a small incision in the suprapubic region and allows us to better observe the growth and metastatic spread of bladder tumor cells growing in their normal *in situ* environment. Additionally, it provides a more physiological approach to evaluate the immune response to bladder cancer cells. Thus, our *in vivo* studies evaluated the use of liposome-mediated, irradiated, gene-modified cells as "tumor vaccines" in orthotopically implanted MBT-2 tumor-bearing animals. Parameters including vaccination schedule and the amount of transfected cells/vaccine were investigated with a final end point of survival. All animals that survived the initial tumor implantation were rechallenged with parental MBT-2 cells. Necropsy and splenic CTL analysis were performed on those animals that survived the rechallenge.

Initially, we studied a vaccination schema based on multiple injections of single-agent gene-modified MBT-2 cells. These included plasmids containing IL-2 and the adhesion molecule B7.1 genes. B7.1 was well characterized by Freeman *et al.* (31) and Chen *et al.* (32) as a T-cell cofactor that is essential for T-cell activation. As the *in vivo* evaluation progressed, we revised our vaccination schedule to determine the effects of using different gene products at different vaccination times within the same tumor-bearing animals. Thus, one animal would receive IL-2 or B7.1 gene-modified cells as a single agent throughout or in combination in an alternating vaccination regimen. These "targeted" sequential vaccinations evolved as an opportunity to stimulate different aspects of the immune response in a time-dependent manner, based on our knowledge of the cascade of events that dictate successful immunomodulation.

MATERIALS AND METHODS

Plasmids and Cell Lines. The pMP6-IL-2 and pMP6-B7–1 plasmids are adeno-associated virus-based plasmids using a cytomegalovirus promoter and including the murine IL-2 or B7.1 genes, respectively. Their constructs have been described previously (33). The MBT-2 murine bladder carcinoma cell line was obtained from Dr. Timothy Ratliff (The University of Iowa, Iowa City, IA) from a transplantable *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide-induced tumor in C3H mice (18). L929 is a well described murine (C3H) fibroblast cell line and was used as a syngeneic control for CTL analysis. Cells were maintained in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine at 37°C and 5% CO_2. MBT-2/IL-2 was a stable retrovirally gene-modified cell line (16, 17). All cell lines were tested for *Mycoplasma* contamination every 6 weeks using a PCR-based assay.

Liposome Preparations and Gene Product Analysis. The liposome used in this study was graciously provided by Vical, Inc. (San Diego, CA). DMRIE/DOPE was composed of positively charged lipid DMRIE in combination with neutral lipid DOPE in a 1:1 molar ratio (27). The lipid reagents were stored at 4°C before use. IL-2 measurement was performed by ELISA (R&D Systems, Minneapolis, MN). B7.1 quantitative analysis was documented by a fluorescence-activated cell sorter using FITC-conjugated rat antimouse B7.1 (PharMingen, San Diego, CA).

Cellular Transfection Procedure. Twenty-four h before transfection, MBT-2 cells were plated on 60-mm dishes at a density of 1×10^6 cells/dish. Various amounts of the liposome (2, 10, 20, 40, 60, 80, 120, and 160 μg) and plasmid DNA (1, 2.5, 5, 7.5, 10, 20, 30, and 40 μg) were diluted separately in serum-free medium to a total volume of 500 μl each and then gently mixed together in polystyrene tubes. This solution was allowed to form complexes at room temperature for 15 min. Adherent MBT-2 cells were rinsed once with serum-free medium. The liposome-DNA complex was then diluted with serum-free medium to a final volume of 4 ml and gently added to the cellular monolayers. After a transfection exposure time

ranging from 15 min to 24 h (0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 12, and 24 h), the transfection solution was rinsed off, and fresh medium with 20% FBS was added. Similar experiments to determine the effect of the growth factor on the transfection process required the addition of 10% FBS during the transfection exposure. In separate studies to determine toxicity, cell counts using trypan blue exclusion were performed 12 h after the completion of exposure to the liposome-DNA complex. Twenty-four h after transfection, all cells were exposed to 70 Gy of irradiation. At the next 24 h time point, all cells were rinsed with PBS and then replenished with serum-free medium. Twenty-four h later, supernatants were collected and frozen at $-20°C$ for future IL-2 ELISA determination. Cell counts were performed at this time to be able to report IL-2 concentrations as ng/10^6 cells/24 h. For time course experiments, *i.e.*, evaluation of the gene product secretion over 30 days, all cells were transfected on day 0 and irradiated on day 1 and then had complete medium replaced every 3 days. Cells were rinsed with PBS and replenished with serum-free medium 24 h before supernatant collection. Different sets of cells were transfected in the same study to evaluate gene product secretion at days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30. Adherent cells at each time point were counted for accurate quantification.

Animal Studies. All animal studies were initiated in 6–8-week-old C3H/HeJ female mice (Jackson Laboratory, Bar Harbor, ME). Freshly prepared MBT-2 cells were implanted orthotopically in the animals. Briefly, animals were anesthetized by an i.p. injection of pentobarbital. Under magnification, a 0.8-cm incision was made transversely in the abdomen, just above the pubis. The bladder was delivered into the surgical field, and the bladder wall was injected with 2×10^4 MBT-2 cells in a total volume of 50 μl of PBS. The wound was closed with surgical staples that were removed on postoperative day 5. All animal studies were overseen and approved by the Research Animal Regulatory Committee advisory groups at both Duke University Medical Center and Memorial Sloan-Kettering Cancer Center.

The date of implantation was designated as day 0. Multiple tumor vaccination schedules included: (*a*) days 1, 2, and 3; (*b*) days 2, 4, and 6; (*c*) days 3, 6, and 9; (*d*) days 4, 8, and 12; (*e*) days 5, 10, and 15; (*f*) days 6, 12, and 18; and (*g*) days 7, 14, and 21. Consecutive tumor vaccinations were accomplished with i.p. injection of 5×10^6 MBT-2 cells in a volume of 100 μl of PBS. Transfected MBT-2 cells were used on posttransfection day 2, which was 1 day after irradiation. Adherent cells were removed from plates with 2% trypsin, washed twice with PBS, and then counted on a hemacytometer. A separate aliquot of cells was replated on 60-mm culture dishes for future determination of gene product secretion. The doses of gene-modified cells for vaccination included 1, 2.5, 4, and 5×10^6 MBT-2 cells. Control animals were injected with 100 μl of PBS.

Daily inspection of animals provided general status, tumor size, and symptoms associated with disease (*i.e.*, hematuria and uremic status). Mice with a tumor burden of more than 10% of body weight or mice presenting cahexic status were sacrificed and necropsied with special attention placed on examination of the lungs, kidneys, bladder, and peritoneal cavity. Animals that survived 60 days were rechallenged by orthotopic implantation of 2×10^4 parental MBT-2 cells, and new control animals also received the same inoculum of MBT-2 cells on the same day. These rechallenged animals did not receive any further vaccinations. On day 120 after the original implantation, or 60 days after rechallenge, all animals were sacrificed and necropsied, and CTL analysis was performed on their splenocytes.

CTL Analysis. Splenocytes were harvested and depleted of RBCs with ammonium chloride Tris buffer. Splenocytes (1.5×10^7) were cultured with 7.5×10^5 stimulator cells (MBT-2/IL-2 cells irradiated at 75 Gy) in 5 ml of Iscove's modified Dulbecco's medium with 10% FBS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 50 μM β-mercaptoethanol per well in a 6-well tissue culture plate. Effector cells were cultured for 5 days at 37°C and 5% CO_2 and harvested for CTL assay.

All 5–10 $\times 10^6$ target cells were labeled with europium diethylenetriamine pentaacetate for 20 min at 4°C. After several washes, 1×10^4 europium-labeled target cells and serial dilutions of effector cells at varying E:T ratios were incubated in 200 μl of RPMI 1640 with 10% heat-inactivated FBS in 96-well V-bottomed plates. The plates were centrifuged at 500 × *g* for 3 min and incubated at 37°C and 5% CO_2 for 4 h. The supernatant (50 μl) was harvested, and europium release was measured by time resolved fluorescence (Delta fluorometer; Wallace, Inc., Gaithersburg, MD; Ref. 34). Spontaneous release was less than 25%. SEs of the means of triplicate cultures were less than 5%.

RESULTS

Liposome-mediated Gene Transfer in Murine MBT-2 Bladder Carcinoma Cells. At first, the critical ratio of liposome:DNA in the DMRIE/DOPE and pMP6-IL-2 transfection was evaluated and proved to be 4:1. Higher ratios of DNA did not improve IL-2 secretion. Twelve h after a 0.25–1-h exposure to DMRIE/DOPE (2–80 μg), there were 88–93% viable MBT-2 cells as evaluated by trypan blue exclusion. Toxicity was observed at 120 and 160 μg of DMRIE/DOPE with 74% and 68% viable cells, respectively. All studies with FBS present during the transfection revealed a 45–80% reduction of gene product secretion as compared with transfection in serum-free medium. The best gene product secretion with the least cellular toxicity was observed at a transfection time exposure to the liposome-DNA complex of 0.5–1 h. Longer exposure led to slightly less IL-2 production, but with higher cellular toxicity. Transfection times less than 0.5 h resulted in a 34–56% decrease in IL-2 secretion. Therefore, the optimal transfection conditions for 60-mm culture dishes, which produced the least toxicity with the greatest IL-2 secretion at the least amount of exposure time, were 40 μg of DMRIE/DOPE/10 μg of pMP6-IL-2 for 30 min in serum-free medium. Using these optimized parameters, IL-2 secretion ranged from 245–305 ng/10^6 MBT-2 cells/24 h, whereas the previously established MBT-2/IL-2 stable transfectant produced IL-2 levels of 1.7–2.2 ng/10^6 cells/24 h.

To determine the extent of IL-2 secretion in this "transient" liposome-mediated transfection, we studied the gene product expression over a 30-day period. All cells were transfected on day 0 according to the optimized conditions as described above. On day 1, cells received 70 Gy of irradiation, and then IL-2 secretion was measured at 3-day intervals (Fig. 1). In three

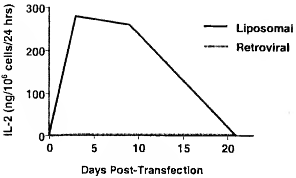

Fig. 1 The IL-2 secretion level from liposome-mediated IL-2 gene-modified MBT-2 cells using 40 μg of DMRIE/DOPE and 10 μg of pMP6-IL-2 plasmid and from stable transfectant MBT-2/IL-2 generated by retroviral transfection was measured over a 20-day time period. All cells were transfected on day 0 and radiated with 70 Gy on day 1. The culture supernatants were collected over a 24-h period and analyzed by ELISA. Ninety percent of the peak IL-2 production from liposome-mediated IL-2 gene-modified MBT-2 cells persisted from day 3 to day 11 after transfection.

separate studies, IL-2 levels reached an average of 280 ng/10^6 cells/24 h by day 3 and maintained 90% of this peak for up to 9–11 days after transfection. A steady decline in IL-2 production was then observed, until levels of less than 5 ng/10^6 cells/24 h were seen beginning on days 15–18. Fourteen to 22% of MBT-2 cells liposomally transfected with B7.1 plasmid expressed cell surface B7.1 as determined by fluorescence-activated cell-sorting analysis.[4]

Effects of Orthotopic Implantation of MBT-2 Cells in C3H Mice. To determine survival, metastatic potential, and tumor growth in the orthotopic bladder tumor model, a series of confirmatory experiments was performed. Animals were sacrificed on days 3, 7, 10, 14, 17, 21, 24, and 28, and total body weight, tumor volume, and tumor weight were measured. Also, metastatic lesions, if present, were inspected. As shown in Table 1, tumor growth was prominent 14 days after tumor implantation, and i.p. metastases were apparent on day 21. These animals used for sacrifice purposes were found to die at nearly the same time as the animals sacrificed because of the humane requirements of the animal committees.

Effects of Liposome-mediated Gene-modified Cells in MBT-2 Tumor-bearing Animals. Mean survival of control animals was 26 days (Table 2). Of all of the vaccination schedules studied, the greatest impact on survival was seen with the day 5, 10, and 15 regimen. Therefore, the group most extensively evaluated was those receiving i.p. vaccinations of irradiated IL-2 gene-modified MBT-2 cells on days 5, 10 and 15 after orthotopic implantation. Initial *in vivo* experiments showed no survival advantage in those tumor-bearing mice that received vaccinations of 1×10^6 IL-2 gene-modified MBT-2 cells. Groups that received vaccinations of 5×10^6 IL-2 gene-modified cells survived for less time than controls, although the

Table 1 Effects of orthotopic implantation of MBT-2 cells in C3H mice

MBT-2 cells (2×10^4) were orthopically implanted in 6–8-week-old C3H mice. Six animals were sacrificed on the days indicated, and total body weight, tumor volume, and tumor weight were measured. Data are a mean of six animals at each time point.

Day	Body weight (g)	Tumor volume (mm³)	Tumor weight (g)	Metastases
3	17.03	NT[a]	NT	No
7	17.59	0.029	0.040	No
10	17.20	0.119	0.130	No
14	17.05	0.180	0.220	No
17	17.36	0.601	0.620	No
21	14.75	0.637	0.731	Peritoneal (4/6)
24	15.69	0.723	0.863	Peritoneal (6/6) Hydronephrosis (4/6)
28	18.28	1.170	1.403	Peritoneal (6/6) Hydronephrosis (4/6) Pancreas (2/6)

[a] NT, no macroscopically identifiable tumor was observed.

Table 2 Comparison of different MBT-2 tumor vaccination doses

All cells were transfected by the optimized liposome-mediated transfection protocol on day 1, irradiated with 70 Gy on day 2, and harvested for vaccination on day 3. Vaccinations were performed on days 5, 10, and 15 after orthotopic implantation of 2×10^4 MBT-2 cells in C3H mice.

	Vaccine	Dose (cells)	Median survival (days)	Range (days)
1	None		26	22–31
2	IL-2[b]	1×10^6	28	22–35
3	IL-2	5×10^6	20	15–25
4[a]	IL-2	1×10^6	27	20–34
	NT	4×10^6		
5[a]	IL-2	2.5×10^6	46	40–52
	NT	2.5×10^6		
6	Retro-IL-2	5×10^6	29	25–34

[a] Groups 4 and 5 represent a combination of IL-2 gene-modified, radiated cells with nontransfected, radiated cells. Group 5 experienced the longest median survival; however, no cures were obtained.

[b] IL-2, IL-2 gene-modified MBT-2 cells; NT, non-transfected MBT-2 cells; Retro-IL-2, retrovirally IL-2 gene-modified MBT-2 cells.

animals were found to have relatively small tumor burdens at the time of necropsy. Most of these animals developed several signs of toxicity (*i.e.*, body weight loss, ascites, chattering, and immobility), and this adverse effect was considered to be IL-2 toxicity because these animals were injected with cells producing >1000 ng of total IL-2 secretion per day. An alternative strategy to increase potential tumor antigen presentation while limiting IL-2 toxicity was devised. This required vaccinations of nontransfected MBT-2 cells in addition to IL-2 gene-modified cells. As shown in Table 2, those animals receiving vaccinations of 2.5×10^6 liposomally transfected IL-2-secreting cells in combination with 2.5×10^6 nontransfected cells repeatedly showed a 75% increase in survival as compared with controls. No other combination of gene-modified and nontransfected cells showed any consistent increase in survival. Also, none of these groups exhibited "cure" or survival for 60 days after tumor implantation. Animals receiving retrovirally gene-modified MBT-2/IL-2 cell preparations had a median survival of 29 days.

[4] W. A. Larchian, unpublished data.

Table 3 Targeted vaccination schema and groups

All cells were transfected by the optimized liposome-mediated transfection protocol on day 1, irradiated with 70 Gy on day 2, and harvested for vaccination on day 3. Vaccinations were performed on days 5, 10, and 15 after orthotopic implantation of 2×10^4 MBT-2 cells in C3H mice.

Targeted vaccination groups[a]		
Day 5	Day 10	Day 15
IL-2	IL-2	IL-2
IL-2	B7	B7
B7	IL-2	IL-2
B7	B7	B7
IL-2/B7	IL-2/B7	IL-2/B7

[a] IL-2, IL-2 gene-modified MBT-2 cells; B7, B7.1 gene-modified MBT-2 cells; IL-2/B7, MBT-2 cells co-transfected with IL-2 and B7.1 genes.

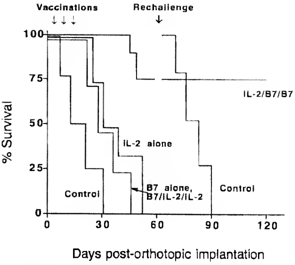

Fig. 2 Targeted tumor vaccinations in the orthotopic MBT-2 bladder tumor model. MBT-2 cells (2×10^4) were orthotopically implanted in all animals on day 0, and animals were vaccinated with i.p. injections of 5×10^6 gene-modified, irradiated cells on days 5, 10, and 15. Ten animals/group were used, and the experiment was repeated twice. Control animals received injections of normal saline. The IL-2 alone, B7 alone, and B7/IL-2/IL-2 groups achieved similar, improved survival, but no cure of cancer was observed. The animals in the IL-2/B7/B7 group had 75% survival on day 60, at which time they were orthotopically rechallenged with 2×10^4 MBT-2 cells, as were new naive controls. No further vaccinations were performed. All surviving animals further survived to day 120. All animals that survived to day 60 survived the tumor rechallenge and were alive at day 120.

Targeted Vaccination Studies in the MBT-2 Model. In an attempt to further modulate the immune response with liposome-mediated gene-modified cells in the MBT-2 tumor-bearing animal model, we evaluated the use of a targeted vaccination regimen. The purpose of these studies was to investigate ways of not only increasing antigen recognition but stimulating the cytotoxic arm of the immune response. Therefore, we initiated experiments evaluating various vaccination schedules using combinations of IL-2 and/or B7.1 gene-modified cells (Table 3). MBT-2 cells were transfected using the described optimized liposome-mediated transfection. The vaccination schedule of day 5, 10, and 15 was followed. Tumor-bearing animals received a vaccination with MBT-2 cells containing one transfected plasmid on day 5, followed by MBT-2 cells that were transfected with another plasmid on days 10 and 15. As seen in the previous experiments, control animals and those receiving IL-2 gene-modified MBT-2 cell vaccinations followed the same course (Fig. 2). In those animals receiving B7.1 gene-modified MBT-2 cells alone or B7.1 followed by IL-2, a survival advantage similar to that seen in the group treated with IL-2 alone was demonstrated. In repeated studies, the group of mice given IL-2-secreting cells on day 5 followed by B7.1-expressing cells on days 10 and 15 showed 75% survival at 60 days. At that time, the survivors were orthotopically rechallenged with MBT-2 cells, as were a new set of control animals. None of the survivors received any further vaccinations during the course of the study. The control mice succumbed as before; however, all of the IL-2/B7/B7 survivors remained alive for another 60 days, or 120 days since the original tumor implantation. Interestingly, a set of animals vaccinated simultaneously with IL-2 and B7.1 cotransfected cells revealed no survival advantage.

All survivors were sacrificed at day 120, and necropsy revealed no evidence of primary tumors or metastases in the chest, peritoneum, or pelvis. As shown in Fig. 3, splenocytes harvested from mice vaccinated with IL-2/B7/B7 exhibited very high levels of CTL activity (approximately 50% at an E:T ratio of 100:1). Control target L929 demonstrated insignificant lysis.

DISCUSSION

The role of immunotherapy in the treatment of cancer is currently being defined. The use of gene therapeutic techniques to transfer cytokine genes into tumor cells has resulted in over 50 new human clinical trials encompassing a variety of malignancies in the past several years (35, 36). Our previous studies in the murine MBT-2 bladder cancer model used the retroviral transfection modality. In this report, we have evaluated an alternative transduction modality, cationic liposomes, in an effort to enhance gene transfer and augment the immune response.

Using the MBT-2 model, we have demonstrated a clear advantage of liposome-mediated transfection over retroviral system. With optimized liposome-mediated transfection, we were able to observe a 100–150-fold increase in IL-2 production in a fraction of the transfection time (30 min) as compared with the retroviral system. This magnitude of the enhanced gene product secretion persisted for over 10–14 days. Although "transient" in a nonintegrated form as episomes as compared with the stable genomically integrated retroviral transfection, IL-2 expression by liposome-mediated gene-modified cells proved to be persistent enough to stimulate an immune response, the ultimate goal of this form of gene therapy. Indeed, these advantages could have a profound impact when considering a human model for cytokine gene therapy. First, the liposome-mediated transfection does not require dividing cells, as the retroviral system does. Therefore, all cells available for transfection would be potential candidates for gene modification strategies. Second, the issue of safety concerning replication defects in the retroviral system is obviated. The safety of liposomes has been confirmed by their use in human trials for the treatment of melanoma (37, 38). In addition, the simplicity and

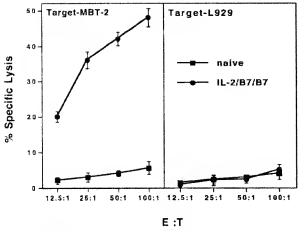

Fig. 3 Priming of CTL responses in mice vaccinated with gene-modified MBT-2 cells. Splenocytes from mice in the IL-2/B7/B7 group, which survived from rechallenge in Fig. 2, were harvested and tested for CTL activity. Splenocytes from naive mice were used as controls. For details, refer to "Materials and Methods." Control target cells (L929 cells) showed insignificant lysis.

virtual instantaneous transfection significantly reduce the laborious and time-consuming efforts necessary for retroviral transfection, both important improvements when considering human application. The 100-fold increase in cytokine production using the liposome technique also has a profound impact in the human gene therapy model. In human trials, we will not have unlimited amounts of primary tumor cells to transfect and use later as tumor vaccines. Therefore, to be able to produce this logarithmically higher cytokine secretion will afford greater potency to each injection of gene-modified cells. Indeed, in human bladder cancer cell lines and in primary cultures of human bladder cancer obtained from surgical specimens, we have been able to achieve similar enhancement of gene expression using liposome-mediated transfection (39).

The orthotopic implantation of bladder cancer in an animal model has proved to be beneficial in allowing the observation of a tumor growing directly in bladder tissue, a more physiological approach to evaluate the immune response to this site. The IL-2 gene-modified tumor vaccines appeared to result in a reliable but limited response. Survival was increased, but no cures were observed. IL-2 has been clearly implicated in the attraction of APCs and consequent stimulation of CD4+ and CD8+ cells. However, we did not achieve a lasting immune response or eradication of the pre-established bladder tumor. The explanation of this successful but limited response may lie in the growing belief that multiple signals and stimulation at various sites of the immunological pathway are essential in promoting a potent and persistent cytotoxic tumor effect (32, 40, 41).

This dictum led to the development of the targeted vaccination studies as described in this report. It is also clear that stimulation of CD4+ and CD8+ cells by professional APCs is required to effect the antigen-specific killing of tumor cells. With this in mind, we attempted to understand and modify our approach to liposome-mediated gene-modified cellular vaccines in the MBT-2 tumor-bearing model.

The inclusion of adhesion molecule B7.1, a T-cell cofactor, in the vaccine regimen was an attempt to investigate alternative modes of activating an immune response. Thus, multiple combinations of IL-2 and B7.1 gene-modified tumor vaccines were tested in the *in vivo* MBT-2 model. The regimen (IL-2/B7/B7) that began with IL-2-secreting cells on day 5, followed by the B7.1-transfected cells on days 10 and 15, had a striking effect on survival. The other groups, which included B7.1 alone (B7/B7/B7) or the alternative schema of B7.1 followed by IL-2 (B7/IL-2/IL-2), did not prove efficacious. So successful was this IL-2/B7/B7 schedule that not only did 75% of the animals experience tumor regression and survive to 60 days after tumor implantation, but all survivors were resistant to orthotopic rechallenge and remained tumor free. The evidence that memory CD8+ response against MBT-2 cells had been achieved in surviving animals is further supported by the CTL analysis (Fig. 3).

Thus far, several previous studies regarding immunogene therapy using B7 and cytokines have shown the controversial results of this strategy in other tumor systems. Gäken *et al.* (42) have successfully shown that combined expression of murine B7.1 and IL-2 on NC murine adenocarcinoma cells was significantly more effective than either B7.1 or IL-2 alone in inducing the immune-mediated rejection of pre-established NC tumors involving CD4+ lymphocytes. Cayeux *et al.* (43) have found that immunization with J558-IL2/B7.1 cells followed by challenge with parental murine plasmacytoma J558L cells caused a reduction in systemic protection as compared with J558-B7.1 or J558-IL2 alone. They concluded that "hyperstimulation" of the immune response by genetically modified cancer vaccines could have adverse effects on tumor immunity. It was also true in our current experiment that simultaneously vaccination with IL-2 and B7.1 cotransfected MBT-2 cells resulted in no survival advantage. The differences in immunological mechanisms are not yet completely understood; however, it is suggested that this difference may be due, in part, to the induction of anergy in the potential reactive T cells.

Our results imply that B7.1 is required for successful induction and function of CTLs after IL-2 exposure. B7.1-transfected MBT-2 cells alone were not responsible for tumor regression when used independently; instead, they appeared to be effective only after the initial activation of the immune response by the IL-2 gene-modified MBT-2 vaccinations. One can therefore speculate that the increased attraction of APCs by IL-2 is the initial complementary step in the cascade of immunological events that subsequently permits the function of B7.1 to be realized as a cofactor in the production and stimulation of antigen-specific CTLs. We believe that this concept of timed, programmed, targeted stimulation of the immune response is a logical alternative to continuous single-agent regimens.

Additional studies involving liposome-mediated gene-modified cellular vaccinations in the MBT-2 bladder cancer model are under way. These will focus on the programmed use of multiple cytokines and T-cell factors in targeted vaccination studies. We are currently investigating the different components of the immune response at each activation step to better identify

the mechanisms involved. In addition, the use of liposome transfection with cytokine genes in human urological malignancies will be actively pursued. This alternative form of cancer treatment, which uses gene transfer techniques to enhance tumor antigen recognition, has multiple applications and holds great promise.

In summary, liposome-mediated transfection provides a safe, simple, and highly effective mode of gene transfer as compared with retroviral transfection systems. The use of multiple agents in gene-modified tumor cell vaccinations in a time-dependent fashion has elicited a more significant and substantial immune response than single-agent regimens.

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